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REVIEW

Leveraging Metabolomics to Assess the Next Generation of Temozolomide-based Therapeutic Approaches for Glioblastomas

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Abstract Glioblastoma multiforme (GBM) is the most common adult primary tumor of the central nervous system. The current standard of care for glioblastoma patients involves a combination of surgery, radiotherapy and chemotherapy with the alkylating agent temozolomide. Several mechanisms underlying the inherent and acquired temozolomide resistance have been identified and contribute to treatment failure. Early identification of temozolomide-resistant GBM patients and improvement of the therapeutic strategies available to treat this malignancy are of uttermost importance. This review initially looks at the molecular pathways underlying GBM formation and development with a particular emphasis placed on recent therapeutic advances made in the field. Our focus will next be directed toward the molecular mechanisms modulating temozolomide resistance in GBM patients and the strategies envisioned to circumvent this resistance. Finally, we highlight the diagnostic and prognostic value of metabolomics in cancers and assess its potential usefulness in improving the current standard of care for GBM patients.

Introduction

Glioblastoma multiforme (GBM) is the most prevalent and aggressive primary brain tumor [1]. It accounts for approxi-

mately 60% of all primary brain gliomas diagnosed yearly in the United States [2]. Although early symptoms associated with GBMs depend on location, size and rate of growth of the tumor, 30–60% of patients experience headaches and seizures [3]. Despite recent progresses in the molecular characterization of GBMs, median survival time of patients suffering from GBMs remains between 12 and 15 months [4,5]. Current standard of care to treat GBM patients consists of surgical resection followed by a regimen that includes radiotherapy plus concurrent and adjuvant chemotherapeutic treatment with temozolomide (TMZ) [6,7]. TMZ is a DNA alkylating agent of the imidazotetrazine class that can effectively cross the blood–brain barrier [8]. Researchers have started to

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uncover mechanisms that underlie TMZ resistance to GBMs. These include the enzyme O⁶-methylguanine-DNA methyltransferase (MGMT) that removes methyl groups from DNA, as well as the DNA mismatch repair cascades capable of repairing mispaired DNA bases [9,10]. Yet, the complete molecular picture associated with TMZ resistance in GBMs remains elusive and the development of novel approaches to characterize the metabolic footprint of GBMs is of great interest. In this review, we present the deregulated pathways involved in GBM formation and progression, focus on the mechanisms underlying TMZ resistance in GBMs and discuss metabolomics-based approaches that could be leveraged in the quest to improve the current therapeutic outcomes in GBMs.

Glioblastomas: a molecular overview

GBMs are grade IV gliomas and arise either *de novo* as primary GBMs or through progressive development from lower grade astrocytomas, which ultimately leads to secondary GBMs [11]. Molecular profiling has revealed deregulated core signaling pathways that confer GBM formation and progression [12]. In addition, a study of gene signatures expressed in GBMs highlighted four distinct subtypes: proneural, neural, classical and mesenchymal [13]. A well-characterized molecular event is amplification of the gene encoding epidermal growth factor receptor (EGFR), which occurs in approximately 50% of primary GBMs [14]. Moreover, 20–30% of GBM patients express a shortened and constitutively active version of EGFR, EGFRvIII, which no longer requires interaction with its ligand EGF to activate downstream signaling cascades such as the PI3K/Akt pathway [15,16]. Genes coding for other receptor tyrosine kinases, including the platelet-derived growth factor receptor (PDGFRA) and the proto-oncogene (MET), are amplified to various degrees in GBMs, resulting in the modulation of proliferative and survival pathways [17,18]. Increased levels of the *MDM2* and *CDK4* oncogenes via chromosome 12q13–15 amplification are also of note in GBMs [19]. On the other hand, mutations of *PTEN* and *p53*, genes coding for two proteins with tumor suppressive capabilities, are frequent occurrences in GBMs [20]. Modifications in *PTEN* and *p53* expression contribute to sustained activation of the PI3K/Akt signaling axis and to evasion of programmed cell death, respectively [21,22]. Furthermore, epigenetic silencing of tumor suppressor genes, including *CDKN2A*, *CDKN2B*, *PTEN*, *RBI* and *p53*, via hypermethylation is common in GBMs [23].

Aside from the gene modulation and epigenetic regulation, the potential implications of microRNAs (miRNAs) in GBMs should not be overlooked [24,25]. MiRNAs are small (18–24 nucleotides) noncoding RNAs that act as post-transcriptional modulators of gene expression and thus play crucial roles in regulating different cellular processes [26]. Several miRNAs with oncogenic potential, or oncomiRs, emerge as underlying drivers of various malignancies including GBMs. miR-26a, which targets *PTEN* and *RB1* tumor suppressors, is frequently coamplified with *CDK4* in GBMs [27]. Expression of miR-21, another known regulator of *PTEN* protein expression, is frequently up-regulated in human GBM samples [28], while antiproliferative effect of miR-21 silencing has been reported in GBM cells [29]. Down-regulation of tumor suppressor miRNAs has also been identified in GBMs.

For example, expression of miR-7, which inhibits EGFR and Akt pathway activities by interacting with key transcript targets within these cascades, was frequently down-regulated in GBMs [30]. Expression of miR-34a, a transcriptional target of p53, is frequently down-regulated in cancer including GBMs. More importantly, expression levels of miR-34a were inversely correlated with protein levels of MET and Notch in gliomas [31].

Overall, either through amplification of selected receptor tyrosine kinases, loss of molecules with tumor suppressive properties or modulation of a family of oncogenic miRNAs, numerous signaling cascades are driving GBMs.

The current standard of care for GBM treatment combines surgical resection, radiotherapy and adjuvant TMZ treatment, leading to increased median survival time [6]. However, the 5-year survival rate remains considerably low either for TMZ treatment combined with radiotherapy or for radiotherapy alone (9.8% vs 1.9%) after surgery [7]. The mechanism of action and challenges associated with this chemotherapeutic agent will be discussed in the next section. Other FDA-approved therapeutic approaches for GBMs include 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) wafers. This method is based on controlled release delivery of carmustine from biodegradable polymer wafers deposited in the tumor cavity upon tumor removal [32]. A meta-analysis of phase III trials revealed that BCNU wafers increased overall survival of primary malignant glioma patients by 2.2 months (13.1 vs 10.9 months) [33]. NovoTTF-100A, a noninvasive electrode system that generates pulsating electric fields and induces apoptosis [34], has been recently approved by FDA. While NovoTTF-100A provides benefits over TMZ treatment including negligible side-effects, a recent study comparing the two approaches indicated that the method was at best comparable to TMZ in terms of survival rate [35]. The monoclonal antibody bevacizumab has also garnered interest as a therapeutic alternative to treat GBMs since its approval in 2009. Bevacizumab, a recombinant anti-VEGF antibody, notably increased progression-free survival and reduced tumor vascularization in GBMs [36]. Nonetheless, bevacizumab does not seem to impact overall survival in these patients either and further trials to evaluate this treatment option are required [36].

Besides the aforementioned FDA-approved therapies, several therapeutic strategies to treat GBM patients are currently being investigated in clinical trials. Such strategies have notably been directed toward differentially expressed or hyperactivated kinases identified in GBMs, such as EGFR [37]. Unfortunately, the effectiveness of small molecule inhibitors of EGFR such as gefitinib and erlotinib has been proved to be highly dependent on PI3K and PTEN status and yielded modest results [16]. PI3K pathway inhibition is an attractive axis in the development of targeted treatments in GBMs. While preclinical studies using PI3K inhibitors have led to promising results [38], clinical trials evaluating enzastaurin, a PKC/PI3K/AKT inhibitor, did not positively impact progression-free survival in GBM patients and were therefore halted [39]. Inhibition of MET, a frequently overexpressed receptor in GBMs, is also currently under evaluation in clinical trials [40]. Unfortunately, a phase II trial using an anti-MET antibody, AMG102, demonstrated no significant antitumor activity in patients with recurrent GBMs [41]. Overall, these therapeutic approaches have yielded at best marginally positive results and TMZ, the hallmark chemotherapeutic agent

routinely used to treat GBMs, remains the primary therapeutic alternative.

Several hurdles need to be overcome to improve the current standard of care offered to GBM patients. An inherent problem associated with GBM treatment remains the blood–brain barrier, which restricts tumor site access for many therapeutic agents [42]. Novel strategies to deliver therapeutic agents to the tumor site are being explored including convection-enhanced delivery, a positive-pressure infusion-based method that can be used to administer chemotherapeutics directly into peritumoral brain [43]. Unfortunately, using small molecular weight inhibitors directed against one molecular target has often led to activation of compensatory signaling pathways leading to treatment failure [44]. Nevertheless, the drawbacks of single-agent therapies provide crucial insight into the ongoing development of combination treatments in GBMs. Simultaneous targeting of key molecular nodes including EGFR, VEGFR, PI3K, CDKs and the JAK/STAT signaling axis has generated promising results in rodent models of GBMs [45–47]. A recent study using a PI3K/mTOR dual inhibitor in human GBM xenografts showed increased survival, when compared to TMZ treatment alone [48]. In addition, clinical trials targeting these molecular nodes are underway to identify sensitizing agents to be used in combination treatments for TMZ-resistant GBM patients [2]. Overall, further investigations are required to address these challenges and improve outcomes for GBM patients.

Temozolomide resistance in glioblastomas

TMZ, the chemotherapeutic agent given as part of the primary standard of care to treat GBMs, is an alkylating agent that adds a methyl (m) group to purine bases of DNA, producing O⁶-guanine (G) (6%), N⁷-G (70%) and N³-adenine (A) (9%) [49,50]. TMZ treatment leads to cell death primarily through O⁶-G methylation [51]. This modification leads to G pairing with thymine (T) during DNA replication and promotes double-stranded DNA crosslinking lesions that are difficult to repair by the DNA mismatch repair system, ultimately contributing to cell death [52,53]. As a lipophilic molecule, TMZ is administered orally and can penetrate the blood–brain barrier with relative ease and has a high bioavailability (>99%) [8,54,55]. TMZ toxicities are typical of an alkylating agent and include hematological side effects such as lymphopenia, thrombocytopenia and leucopenia [6].

Unfortunately, inherent and acquired TMZ resistance is a common occurrence in GBM patients. Such resistance in gliomas is strongly correlated with the presence and activity status of the DNA-repair enzyme O⁶-MGMT, an enzyme capable of removing methyl groups from the O⁶ position of G residues and counteracting the cytotoxic effects of TMZ [9,56]. MGMT protein expression in GBM tumors can significantly increase their ability to resist TMZ treatment [57,58]. MGMT expression is reduced by hypermethylation of its promoter, resulting in increased TMZ sensitivity [59]. MGMT hypermethylation is notably detected in 45–70% of high grade gliomas [60].

Other factors, besides MGMT expression, can contribute to TMZ resistance in GBMs. A functional DNA mismatch repair system is required for TMZ sensitivity. This pathway recognizes the O⁶-mG-T mismatch and recruits proteins that excise specifically the erroneous T thus recycling the original

O⁶-mG. O⁶-mG is subsequently mispaired with another T and the adduct is repaired creating a cycle that ultimately leads to persistent DNA breaks, cell cycle arrest and apoptosis [61,62]. A deficient DNA mismatch repair system can thus contribute to TMZ resistance. Expression of mismatch repair protein MSH6 is down-regulated in GBM patients treated with TMZ, which could play an influential role in acquired resistance to the drug [63]. While TMZ cytotoxic effects are primarily attributable to the O⁶-mG lesion, the N⁷-mG and N³-mA modifications cannot be overlooked. Components of the base excision repair pathway rapidly remove and repair the modified bases and contribute to TMZ resistance. AP endonuclease (APE-1), a key enzyme in this pathway, is linked to TMZ resistance due to its up-regulated expression in human gliomas [64]. Interestingly, inhibition of base excision repair pathway sensitized cells to TMZ in ovarian cancer via increased cytotoxic effects of N³-mA and N⁷-mG [65].

Looking ahead, improving TMZ sensitivity in GBM patients is conceivable and might require undertaking multi-targeted therapeutic approaches directed at the aforementioned repair mechanisms or modifying the TMZ molecule itself. Inhibition of the base excision repair pathway can sensitize GBM cells to TMZ [66]. Pharmacological inhibition of APE-1 with small molecule inhibitors in preclinical models potentiated the cytotoxicity of alkylating agents [67]. Inhibition of poly(ADP-ribose)polymerase (PARP), an enzyme involved in the DNA repair pathway, also improved TMZ sensitivity in various models *in vitro* and *in vivo* [68]. Structurally, TMZ is an imidazotetrazine that can deliver methyl groups to selected DNA bases. Synthesis of imidazotetrazine analogues capable of adding a chemical group unrecognizable by MGMT could potentially circumvent the basic repair mechanisms underlying TMZ resistance. A series of such analogues were recently tested for their cytotoxic effect in TMZ-resistant GBM cells and two lead molecules with anticancer properties irrespective of MGMT and DNA mismatch repair pathway status were identified [69]. Unfortunately, the lead compounds identified in this study also demonstrated significant plasma instability in a mouse model thus raising doubts on their *in vivo* usefulness. While multi-targeted approaches to sensitize GBM cells to TMZ and improvement of the drug itself are of interest, a better characterization of the molecular footprint associated with TMZ resistance is needed for such strategies to succeed.

Metabolomics as a tool for cancer research

Metabolomics and metabonomics, provide the quantitative measurement of metabolic composition as well as metabolic changes that occur in living systems as a result of a pathophysiological stimuli or genetic modification. Metabolomics (in this text used to represent both metabolomics and metabonomics approaches) can provide a snapshot of the biochemical pathways modulated under different conditions [70]. It measures the collection of all small molecule metabolites or chemicals that can be found in a cell, organ or organism [71]. This metabolic profile, the metabolome, can be leveraged for different purposes. This section focuses on the usefulness of metabolomics in cancer diagnosis, prognosis and therapeutic response assessment with a special emphasis on GBMs.

Various metabolic changes are at work in cancer cells initially due to the functions of oncogenes and oncosuppressors

Table 1 Differentially expressed metabolites with diagnostic significance in selected brain tumors

Metabolites	Sample type	Tumor samples	Sample size	Method	Ref
↑Choline, ↓NAA	<i>In situ</i>	Gliomas vs. non-neoplastic lesions	28	H-MRS	[88]
↑Choline, ↓NAA, ↓creatine,	<i>In situ</i>	Gliomas vs. non-neoplastic lesions	164	H-MRS	[87]
↑Alanine, ↑valine, ↓proline, ↓glutamate, ↓glutamine, ↓GABA, ↓NAA	Primary tissue samples	High-grade vs. low-grade oligodendrogliomas	34	HR-MAS	[93]
↑Taurine, ↑GPC, ↑P-choline, ↑choline, ↓NAA, ↑myo-inositol	Intact tissue samples	Medulloblastomas vs. ependymomas and pilocytic astrocytomas (all pediatric)	20	HR-MAS	[85]
↑Fatty acids, ↑isoleucine, ↑leucine, ↑valine, ↑NAA	Intact tissue samples	Pilocytic astrocytomas vs. ependymomas and medulloblastomas (all pediatric)	20	HR-MAS	[85]
↓Myo-inositol	<i>In situ</i>	GBMs vs. low-grade astrocytomas	39	H-MRS	[91]
↑Lactate	<i>In situ</i>	High-grade vs. low-grade gliomas (WHO grades 2 and 3)	213	H-MRS	[86]

Note: GABA, γ -aminobutyric acid; GBM, glioblastoma multiforme; GPC, glycerophosphocholine; NAA, N-acetyl-aspartic acid; P-choline, phosphocholine; H-MRS, proton magnetic resonance spectroscopy; HR-MAS, high-resolution proton magnetic angle spinning spectroscopy; WHO, World Health Organization.

and are subsequently promoted by changing cellular environment [72,73]. The highly proliferative status of cancer cells translates into elevated energy and biomaterial requirements and leads to increased consumption of some metabolites such as glucose and glutamine, altered energy generation and changes in biomaterial generation routes [74,75]. Increased glycolytic capacity and elevated phospholipid levels have been reported in several cancer models [76]. In contrast, metabolites such as amino acids and nucleotides have different signatures, depending on the cancer type assessed [77]. Metabolomics, as a cancer diagnosis tool, can help in characterizing differentially expressed metabolites between normal and cancer cells or between cancer subtypes or stages. Metabolomics analysis of tissue *ex vivo* as well as *in vivo* can provide clear distinction between tumor and healthy cells and can be used in diagnosis of many tumor types. A study comparing the metabolome of breast cancer samples and normal specimens identified the malignant samples with considerable sensitivity and specificity [78]. Using a similar approach, elevated levels of taurine, lactate and choline were also detected in colorectal cancer tissue specimens [79]. While unlocking the metabolome of a primary tumor can yield interesting insights into the differentially regulated pathways underlying the malignancy, assessing circulating metabolites in cancer patients also holds tremendous diagnostic potential albeit with still outstanding issues regarding confounding factors. A metabolomics-based approach was employed to characterize the profile of circulating metabolites in epithelial ovarian cancer patients and was able to discriminate between cancer patients and healthy premenopausal subjects [80]. A recent study identified 22 differentially expressed metabolites in the urine of epithelial ovarian cancer patients versus healthy individuals [81]. The metabolic signatures of urine samples collected from esophageal cancer patients demonstrated a distinctive footprint that allowed discrimination between esophageal carcinoma and healthy controls as well [82]. Similarly, several metabolomics-based approaches have been undertaken in gliomas [83]. Choline, lactate and glutamine were able to differentiate between GBM cell lines [84]. In primary tumors of pediatric origin, phosphocholine was identified as a potential differentiator between medulloblasto-

mas, ependymomas and pilocytic astrocytomas [85]. Moreover, levels of lactate and lipid could assist in differentiating low-grade from high-grade primary gliomas [86]. Similarly, another research group demonstrated that gliomas of higher grade exhibited significantly elevated choline levels and increased lipid synthesis [87]. In biopsies obtained from different brain mass lesions, increased choline levels and decreased N-acetyl-aspartic acid (NAA) levels were indicators of tumorigenic samples [88]. Choline is an intermediate of phospholipid metabolism and serves as an important building block for synthesis of selected lipids required for cell membrane structure and function [89]. As a result, elevated choline levels are needed in conditions of increased cell-membrane turnover such as in proliferating cells [90]. Lower myo-inositol levels were reported in GBMs, when compared to low-grade astrocytomas [91]. This finding is aligned with a previous report that described myo-inositol as a molecule primarily located in astrocytes [92]. In addition, alanine and valine were capable of assisting with the grading of oligodendrogliomas [93]. Interestingly, this study demonstrated increased levels in high-grade oligodendrogliomas of these two amino acids, which were linked to anaerobic metabolism, along with a concurrent reduction in molecules related to the Krebs pathway, such as proline, glutamate, glutamine and NAA. It was hypothesized that this metabolic shift toward fermentative metabolism was indicative of tumor hypoxia in high-grade oligodendrogliomas. Metabolites isolated from cerebrospinal fluid (CSF) of glioma patients revealed a distinctive metabolic signature, when compared with samples of healthy controls [94]. A summary of key findings on differentially expressed metabolites in gliomas is presented in Table 1.

Besides its diagnostic utility, metabolomics also holds tremendous potential as a tool to monitor treatment response in cancer patients. Several studies highlighting this application have notably been conducted in breast cancer patients. Analysis of the metabolic profiles of serum collected from metastatic breast cancer patients assisted in identifying a subset of patients that were more likely to respond to combination therapy [95]. Four metabolites including threonine, isoleucine, glutamine and linolenic acid were also found to serve as good pre-

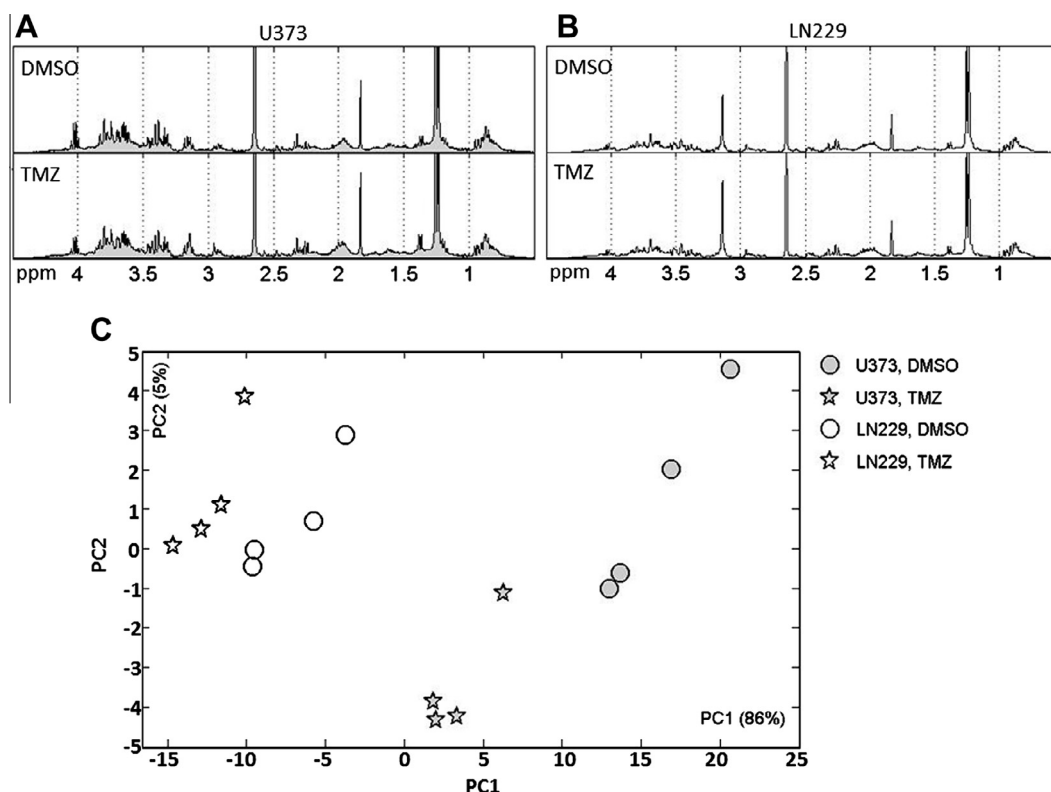


Figure 1 Metabolomics analysis of hydrophilic metabolites extracted from GBM cell lines

NMR profiles of hydrophilic metabolites extracted from TMZ-sensitive (U373) (A) and TMZ-resistant (LN229) (B) GBM cell lines were obtained for TMZ-treated and control lines. C. Principal component analysis (PCA) of metabolic profiles indicates that PC1 summarizes 86% of variances in the data and shows changes in different sample groups. Further analysis of specific metabolic differences is possible from NMR data, possibly leading to markers for TMZ response and treatment follow-up.

dictors of neoadjuvant chemotherapeutic response in breast cancer patients [96]. The potential importance of choline-containing compounds as a biomarker for therapeutic response in cancer has also been proposed. Down-regulation of such compounds was associated with a positive therapeutic response in breast, prostate and brain cancer [97]. Studies using metabolomics to monitor and predict therapeutic response in GBM patients are sparse, yet this application is of great interest. Metabolic assessment of extracellular fluid collected in GBM patients that undergo conventional radiotherapy highlighted the potential of detecting metabolic markers for the prediction of early treatment response [98]. With the inherent challenges that exist with the current therapies available to treat GBMs, a noninvasive tool for early prediction of TMZ response would hold great promise. Primary analysis of metabolic profiles by nuclear magnetic resonance (NMR) spectroscopy in a TMZ-resistant and a TMZ-sensitive GBM cell line shows clear differences (Figure 1). NMR spectroscopy provides highly reliable measurements of metabolic profiles in any biological system. Spectral data can be used directly for the analysis of metabolic differences between cell lines using principal component analysis (PCA). PCA evaluation of U373 and LN229 cells treated with 250 μ M TMZ or vehicle clearly depicted differences in metabolic profiles in two cell types as well as changes in metabolic profiles following TMZ treatment (St-Coeur et al., unpublished data). This indicates potential for utilizing metabolomics for prediction of tumor response to TMZ. Nonetheless, much remains to be done, such as comparing

the quantitative metabolic profiles of various TMZ-resistant GBM cell lines with similar samples collected from TMZ-sensitive cell models as well as analysis of metabolic response to TMZ treatment in these distinct cell types. This process will subsequently need to be validated in clinically relevant samples such as primary GBM tumors or serum collected from GBM patients, thus striving toward the identification of metabolic markers for TMZ treatment planning.

Outlook

GBMs are aggressive brain tumors for which therapeutic alternatives are limited. In addition, the chemotherapeutic agent used as part of the current standard of care is linked to inherent and acquired resistance, which often leads to treatment failure. Looking ahead, rational design of modified alkylating agents using TMZ as scaffold and combinatorial therapeutic approaches are envisioned to improve the current prognosis for GBM patients. It is expected that identification of metabolic markers via metabolomics-based tools, whether to discriminate between specific tumor subtypes or to assist in predicting treatment response, will be of great help in the management of GBMs.

Competing interests

The authors declare no conflict of interests.

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